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Photodynamic Therapy of U87 Human Glioma in Nude Rat Using Liposome-Delivered Photofrin

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Background and Objective: **Liposomes as photosensitizer carriers may enhance the photodynamic effect on tumors.**
Study Design/Materials and Methods: **To test this hypothesis, we treated U87 human glioma in rat brain with photodynamic therapy (PDT) using Photofrin encapsulated in a liposome carrier or Photofrin in dextrose. Nontumored brain was also treated and Photofrin content ratios were measured in tumor and nontumored brain.**
Results: **PDT using the liposome encapsulated photosensitizer significantly increased tumor destruction compared to PDT with Photofrin in dextrose ($P = 0.007$), whereas no difference in tissue damage was detected in nontumored brain with or without liposome carrier. Photofrin uptake was also significantly elevated in the liposome vehicle group compared to the dextrose ($P < 0.05$) group.**
Conclusions: **Our data suggest that Photofrin encapsulated in a liposome may enhance the PDT treatment of human brain tumors. Lasers Surg. Med. 22:74–80, 1998.** **© 1998 Wiley-Liss, Inc.**

Key words: human glioma; liposome; nude rat; PDT; Photofrin

INTRODUCTION

Primary central nervous system malignancies, most of which are gliomas, account for ~11,000 deaths annually [1]. Glioblastomas (or high-grade astrocytomas) constitute the third highest (2.5%) cause of cancer mortality in the 15-to-24-year-old age group [2]. These tumors often recur within 6 months of surgery, radiotherapy, or chemotherapy, and 80% of these patients die within a year [3,4].

Photodynamic therapy (PDT) utilizes a photosensitizer that is selectively taken up and or retained by neoplastic tissue. When absorbing light of an appropriate wavelength, the photosensitizer produces cytotoxic oxygen products (5) causing direct cell death, and/or vascular shutdown. Photofrin has been employed as a photosensitizer for PDT in clinical [6] and experimental animal studies [7–9] of brain tumor.

Phase I and II trials of Photofrin mediated PDT in humans with gliomas have been conducted [10]. Muller and Wilson [11] have employed a laser-coupled inflatable balloon containing a lipid solution to treat patients. The balloon fills the empty tumor crater and serves as a light focusing reservoir to deliver isotropic light energy to the brain adjacent to tumor (BAT). The morbidity associated with their procedure is low [11].

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Muller and Wilson [6] have recently reported a larger series of 56 patients with recurrent gliomas whom they treated with subtotal resection and phototherapy using either Photofrin I (hematoporphyrin derivative), or Photofrin II (dihematoporphyrin ether) as photosensitizers. Overall survival was prolonged with this regimen [6]. Kaye and colleagues [10,12] have treated hundreds of human cerebral gliomas over the years using PDT. The vast majority of studies in humans have used cavitary PDT postresection to treat the region surrounding the removed tumor [10,12]. Local recurrence has been prevented in a high number of cases [10,12].

A role for PDT in the management of deep-seated solid tumors that are not candidates for other modalities has been demonstrated [13]. In a study of eight patients, Powers used interstitial PDT and clearly demonstrated both functional and radiographic improvement in outcome [13]. However, PDT remains an adjuvant brain tumor treatment in humans at present [6,10–13].

PDT with Photofrin as the photosensitizer has been employed to treat 9L gliosarcoma in the rat brain [7,8] as well as the U87 human glioma in the nude rat model [9]. Although experimental brain tumors respond to PDT with Photofrin and tumor destruction correlates with increasing light and photosensitizer doses, normal brain may be affected [14], limiting the efficacy of this treatment modality. It is important to enhance the selective sensitivity of brain tumor to PDT compared to PDT of normal brain. To facilitate preferential brain tumor destruction by PDT, the photosensitizer Photofrin, encapsulated in a dipalmitoyldiphosphatidyl-choline (DPPC) liposome has been employed [8]. Treatment of the 9L gliosarcoma in the Fischer rat following administered Photofrin in a liposome complex significantly increased tumor destruction by PDT compared to Photofrin administered in a dextrose solution. The aim of the present study is to test the tumorcidal activity of PDT in the U87 human glioma in the nude rat brain using Photofrin encapsulated in a liposome.

MATERIALS AND METHODS

Cell Culture

U87 is a human glioblastoma cell line that has been employed in both in vitro and in vivo studies of cancer therapy [15–17]. U87 cells are highly malignant, especially in nude mice and rats [15]. U87 cells were kept in monolayer culture (37°C, 5% CO2) in minimum essential media (MEM) with Earle's salts supplemented and 10% fetal bovine serum, 0.2% phenol red, and 1% each of l-glutamine, MEM sodium pyruvate, nonessential amino acids, penicillin, and streptomycin (Gibco, Grand Island, NY). Cells were subcultured and used for implant when in the exponential growth phase. For harvest, cells were incubated 5 min with 0.05% trypsin EDTA (0.53 mM, Gibco), followed by MEM addition to make a single cell solution.

U87 Implants

Athymic (nude) rats (strain Cr: NIH-rna) were obtained from the National Cancer Institute (Frederick, MD). Sterile techniques were used throughout. Nude rats (90–110 g) were implanted with U87 cells (500,000 cells/5 μL) through a 5 mm craniectomy over the right hemisphere, anterior to the coronal suture. Cells were slowly injected into the cortex 2.5 mm deep, 3.0 mm to the right, and 2.5 mm anterior to the bregma using a 10 ml Hamilton syringe. The syringe was slowly withdrawn after 5 min, the craniectomy covered with polyvinyl chloride (PVC) film glued to the surrounding intact bone, and the incision closed with 4-0 silk suture (Ethicon, Somerville, NJ).

Preparation of Photofrin-Liposome System

Small unilaminar vesicles of DPPC containing Photofrin were prepared immediately before use as previously described [8]. Briefly, DPPC was purchased from Sigma (St. Louis, MO), and Photofrin was obtained from QuadraLogic Technologies (Vancouver, BC, Canada). The liposome was prepared by dissolving 51.4 mg of DPPC in 10 ml of 12.5 mg/ml Photofrin solution in chloroform-methanol (9:1). After thoroughly mixing for 30 min, the solvent was removed by vacuum rotary evaporation at 30°C. The solid was resuspended in 10 ml 0.01 M phosphate buffer at pH 7.4, containing 150 mM NaCl. The solution was then sonicated for 30 min at 50°C. Following this procedure, the final solution contained unilaminar liposomes, which remain stable for ~48 h if refrigerated under nitrogen.

Light Delivery

An argon-dye laser system (Coherent, model INNOVA-70 and CR-500, Palo Alto, CA) provided the light (630 nm) for the PDT treatment and optical measurement. The light was coupled into a 400 μm optical fiber with a distal microlens (PDT,

Santa Barbara, CA) for a 5 mm diameter, uniform spot for superficial irradiation. The power at the distal end of the fiber was adjusted to 20 mW and was measured before and after each treatment with a power meter (Photodyne, Westlake Village, CA) with a 1″ integrating sphere detector head. The irradiation power proved stable in all experiments.

PDT Treatment

On day 14 posttumor implantation, Photofrin dissolved in dextrose vehicle (4 rats) or in liposome vehicle (5 rats) was injected via the femoral vein at a dose of 12.5 mg/kg. Animals were treated with photo illumination 24 h later (15 days after tumor implantation). Animals were anesthetized with ketamine (80 mg/kg) and xylazine (13 mg/kg). Surgery and light treatment were performed using aseptic technique. The rat core body temperature was monitored and maintained at 37°C using a rectal thermocouple with a feedback mechanism controlling a water recirculating module. Laser light was delivered through the 5 mm craniectomy after the PVC film was removed. In a separate nontumor-bearing rat population, normal brain was treated in the same manner, with the craniectomy performed at the time of light treatment. A total optical energy dose of 17 J/cm^2 was delivered.

Histopathology

Animals were sacrificed 24 h post-PDT under anesthesia with ketamine (80 mg/kg) and xylazine (13 mg/kg) administered im. The animals were perfused via the left ventricle with neutral-buffered formalin following vascular washout with heparinized saline. The brain was removed and the brain tissue, encompassing the entire tumor, was cut into four (3 mm thick) coronal blocks. Tumor-bearing brain sections were processed, placed in paraffin, and subsequently five equally spaced sections (6 μm thick) were obtained from each of the 3 mm thick blocks encompassing the tumor. The sections were stained with hematoxylin and eosin (H&E) for light microscopic examination and analysis. The volume of tumor tissue damaged by the PDT was measured by an observer blinded to the treatment conditions, using light microscopic analysis and the Global Lab Image analysis program (Data Translation, Marlboro, MA).

Photofrin Concentration Measurement

Sixteen days after tumor implantation, two separate groups of animals (4 animals each) were injected (iv) at a dose of 12.5 mg/kg with either Photofrin-dextrose or Photofrin-liposome solution, respectively. Twenty-four hours postinjection, animals were anesthetized with ketamine (80 mg/kg) and xylazine (13 mg/kg) and decapitated. The brains were quickly removed and tumor, brain adjacent to tumor (BAT), and the contralateral nontumor containing hemisphere were dissected and frozen at −70°C. Ex vivo tissue Photofrin quantification was performed as described previously [18].

Briefly, tissue samples of ~0.1 g wet weight (Wt) were immersed in 2 ml of Solvable (Dupont-Biotechnology Systems, Boston, MA) and placed into a water bath at 50°C for 1 h, followed by mechanically homogenization (Tissue Tearor, Biospec Products, Racine, WI) of the sample. Up to five 200 μl aliquots of each sample homogenate and an equal number of aliquots spiked with a known amount of Photofrin (V_{sp} = 50 μl, C_{sp} = 37.5 μg/μl), were each diluted with 1 ml Solvable and 3 ml distilled water and returned to the water bath for another hour. The Photofrin used to spike the solubilized samples was monomerized by diluting a stock solution with methanol (0.075 mg/ml) with further dilution to C_{sp} using distilled water. The optical density (OD) of all aliquots was measured in a double-beam absorption spectrophotometer (Shimadzu, Mandel Scientific Co., ON, Canada), and, if required, these aliquots were diluted with double-distilled water to achieve an OD < 0.1 per 10 mm pathlength. For analysis, the aliquots were excited by 405 nm light and the fluorescence intensity recorded. The porphyrin emission peaked was integrated in the 590–730 nm range.

RESULTS

The concentrations and standard deviations of Photofrin in the tumor, contralateral hemisphere, and control tissues from all animals are summarized in Table 1. In both the Photofrin-liposome and Photofrin-dextrose injected animals, Photofrin concentration was significantly higher in the U87 glioma than in the contralateral normal brain tissue or BAT. Photofrin concentration in U87 glioma with DPPC liposome delivery was significantly higher (P = 0.029) than in animals with dextrose delivery. However, the Photofrin concentration in BAT and normal brain remained unchanged. This results in an increase of ~2.4 in the specific uptake of Photofrin-DPPC-liposome in the U87 glioma.

TABLE 1. Liposome—i.v.—U87: Tissue Concentration of Photofrin (μg/g)

Tissue type	Photofrin-liposome (n = 4) tissue concentration ± SD	Photofrin dextrose (n = 4) tissue concentration ± SD
Tumor	8.57 ± 1.16*	3.62 ± 3.03**
Brain adjacent to tumor	0.75 ± 0.24	0.75 ± 0.11
Normal brain	0.42 ± 0.18	0.47 ± 0.13

*$P < 0.05$ compared to the tumor in the Photofrin-dextrose animal, $P < 0.001$ compared to brain adjacent to tumor and normal brain in Photofrin-liposome animals.
**$P < 0.05$ compared to brain adjacent to tumor and normal brain in Photofrin-dextrose animals.

Effect of Photodynamic Therapy on Intracerebral Tumor

At day 16 after U87 human tumor implantation, a uniform tumor was clearly defined by H&E staining under microscopic histological analysis (Fig. 1). At the chosen treatment parameters, the PDT induced tumorcidal activity was intense in the Photofrin-liposome treatment animals and only slight in the Photofrin-dextrose treatment group animals (Fig. 2a–d). In Photofrin-dextrose PDT-treated tumors, a few individual tumor cells exhibited necrotic morphology attributed to PDT. However, under the same experimental conditions, Photofrin-liposome PDT-treated tumors exhibited severe damage. Hemorrhagic foci and necrosis were present. Necrotic tumor was most prominent in areas where the optical radiation intensity was highest. The total % volume of the necrotic tumor tissue was 18.1% ± 3.7 (M ± SD) for Photofrin-liposome PDT compared to only scattered cellular necrosis for Photofrin-dextrose PDT ($P = 0.007$).

PDT-induced lesions in normal rat brain are not significantly different for the dextrose vehicle and liposome vehicle groups at the chosen treatment parameters. Figure 3a,b illustrates the effect of PDT treatments on normal brain by the extent of the H&E staining. Large vessel (>30 μm) occlusion in normal rat brain could not be demonstrated in any rats, but isolated, multifocal capillary, and venule plugging by unidentified material was evident in all animals.

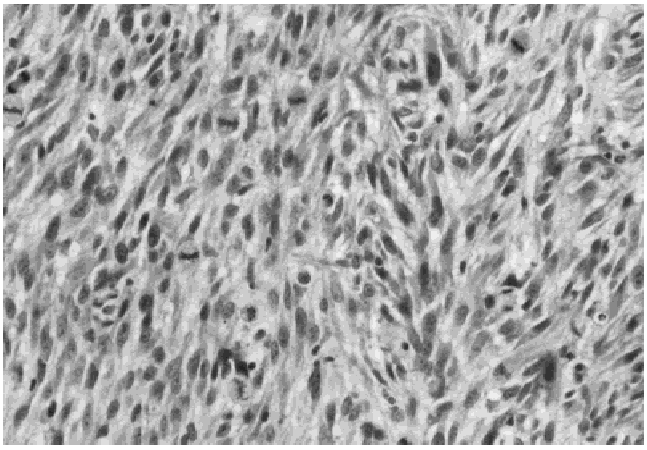

Fig. 1. A paraffin section from an untreated U87 tumor-bearing rat brain, showing nuclear angulation, hyperchromatism and pleomorphism at day 16 after tumor implantation. A common cell variant is formed of mitotically active cells with hyperchromatic elongated (arrows). H&E stain, ×130.

DISCUSSION

Our data indicate that PDT using Photofrin in a liposome vehicle causes a significant increase in U87 glioma brain tumor destruction compared to PDT using Photofrin in a dextrose vehicle. The enhanced treatment of the human U87 glioma with Photofrin-liposome PDT may be attributed to the increased Photofrin concentration in the tumor tissue using a model of tissue response to PDT such as the photodynamic threshold model [19].

The uptake, given by tissue concentration (tumor, BAT, normal brain) per injected Photofrin dose, is lower in this rat model compared with the clinical studies of Muller and Wilson [6], as well as the preclinical rabbit studies by Lilge et al. [14]. However, for the Photofrin-dextrose delivery, the specific uptake ratio, given by tumor tissue concentration per normal brain tissue concentration, is comparable to the ratios in Muller and Lilge's work [6,14]. The specific uptake ratio for Photofrin-DPPC liposomes is significantly higher than that for Photofrin-dextrose delivery in our present study.

The final concentration of Photofrin in liposome was not measured in our study. Photofrin contains at least 85% porfimer sodium, and the rest are monomers, which are not localized in tissue (Dr. Wally Li, Quadralogic Technologies, pers.

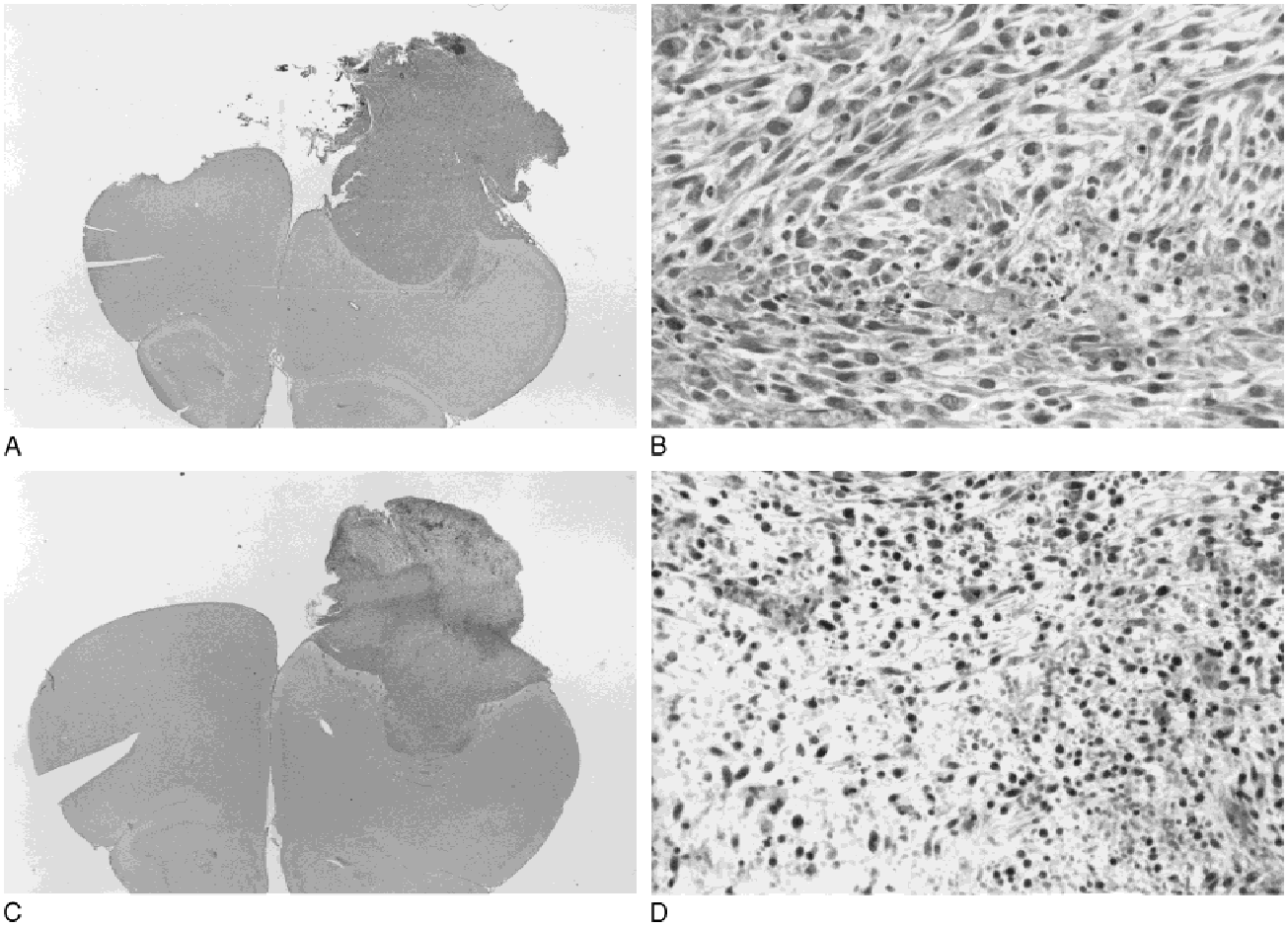

Fig. 2. Paraffin sections from U87 tumor-bearing rat brains, showing a tumor response at 24 h after PDT with an incident energy dose level of 17 J/cm^2. Histopathological examinations reveal that U87 tumor-bearing rats subjected to Photofrin i.v. at a dose of 12.5 mg/kg dissolved in a dextrose vehicle: **(A)** small foci of necrosis and hemorrhage are localized to the superficial area where the intensity of irradiation is the greatest, and **(B)** a high magnification of regions from (A). In a DPPC liposome vehicle: **(C)** coagulation necrosis and hemorrhage with tumor debris of breakdown cellular structures are present with the tumor, and **(D)** a high magnification of regions from (C). H&E stain, a,c. 16×; b. 130×; d. 80×.

comm.). It is likely that in vivo PDT activity of Photofrin encapsulated in liposome results from the retention of only the oligomer fraction in the liposome, since monomers easily wash out.

Liposome encapsulation may preferentially localize to different intracellular sites than Photofrin in dextrose. Photodynamic damage *may* take place on membranes, cytoplasmic enzymes, mitochondria, lysosomes, microsomes, and nuclei [20–22]. In an in vitro study of PDT with porphyrin encapsulated in liposome, Ricchelli et al. [23] noted porphyrin localization in mitochondria and not in plasma membranes. Porphyrin affinity for mitochondrial membranes is highly dependent on the attached side groups of the porphyrin ring itself [20,21]. The study of Woodburn and Kaye [21] on porphyrins shows a steady correlation between the degree of phototoxicity and the extent of localization of porphyrins to the mitochondrial membranes as determined by laser confocal microscopy [21]. The lysosomes were the second most common target for porphyrins [21]. As the side groups synthesized on the porphyrin ring become more cationic, the likelihood of mitochondrial localization increases [21].

Liposomal vesicles have been extensively used as simplified model membranes: the coexistence of amphophilic phospholipid bilayers and aqueous compartments provides a microheterogeneous system that mimics the membrane structure. Plasma membranes exhibit a remarkable increase in binding when hematoporphyrin is transported by DPPC liposome compared to dextrose and water dissolved drug [24]. Thus liposome can

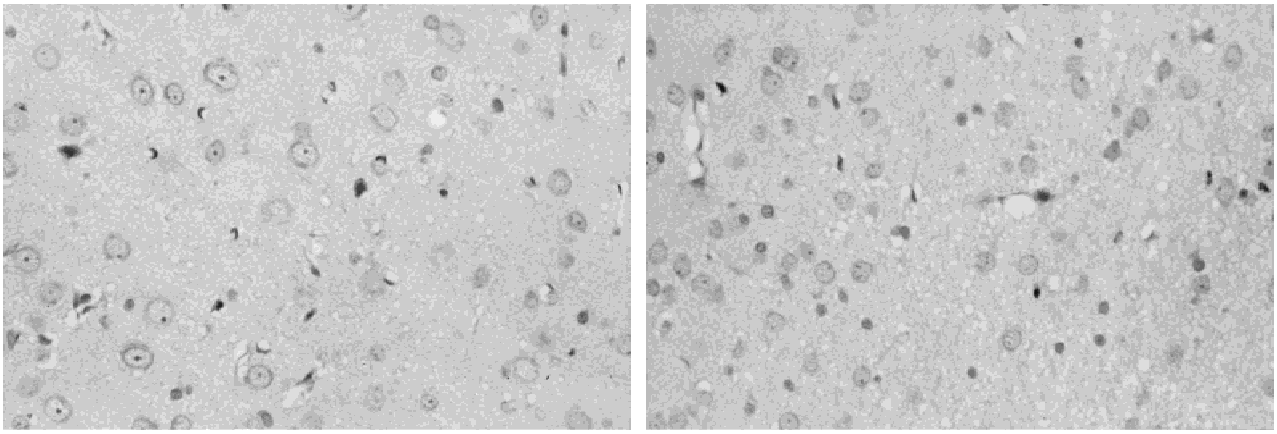

Fig. 3. Paraffin sections from normal cerebral cortices in nontumor-bearing rat brain, showing lesions generated at 24 h after PDT with an incident energy dose level of 17 J/cm^2. Scattered necrotic neurons (pyknotic nuclei and eosinophilic cytoplasm) are observed in rats subjected to treatment with either **(A)** Photofrin-dextrose vehicle, or **(B)** Photofrin-liposome vehicle at a dose of 12.5 mg/kg 24 h prior to laser treatment. H&E stain, 260×.

localize and therefore target Photofrin to different cellular and subcellular membranes. Further studies measuring the subcellular localization of Photofrin-liposome and Photofrin-dextrose are therefore warranted.

This study is an attempt to increase the therapeutic efficacy of PDT for cranial tumors. The U87 human glioma response to PDT with Photofrin delivered by liposome is similar to that measured in the 9L brain tumor under the same conditions [8]. Hence, the data indicate that changing the drug delivery system of Photofrin from dextrose to DPPC liposome may also enhance the human brain tumor response to PDT.

In this study, we selected an i.v. route of administration of Photofrin-liposome. The hydrophilicity of Photofrin may result in a rapid release of Photofrin from the liposome complex after injection, and therefore i.p. injection could affect blood circulation kinetics and alter tissue distribution. However, our data indicate that the tumor response to PDT with Photofrin by i.v. administration is comparable to that with Photofrin-liposome administered i.p. [8], suggesting a sufficiently long in vivo half-life of the Photofrin-DPPC-liposome complex.

PDT of brain tumor with the photosensitizer encapsulated with a liposome may find clinical application. We have demonstrated an enhanced sensitivity of human glioma to PDT using a liposome vehicle. We may be able to capitalize on the liposome-mediated increase in therapeutic index by reducing the optical fluence or Photofrin dose delivered to a tumor-debulked brain. Such reductions may reduce adverse side effects of edema and nontumor tissue destruction.

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